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Cytohesin 2/ARF6 regulates preadipocyte migration through the activation of ERK1/2

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ABSTRACT

Preadipocyte migration is vital for the development of adipose tissue, which plays a crucial role in lipid metabolism. ADP-ribosylation factor 6 (ARF6) small GTPase, which regulates membrane trafficking, is activated by GTP-exchange factors (GEFs) such as cytohesin 2. Cytohesin 2 and ARF6 have previously been implicated in the regulation of 3T3-L1 preadipocyte migration. We investigated here the molecular mechanism underlying the cytohesin 2 and ARF6 mediated regulation of preadipocyte migration. Preadipocyte migration and the activation of ARF6 and ERK1/2 were studied by using a number of approaches, including pharmacological inhibitors, siRNA and the inhibitory peptides. The siRNA mediated down regulation of ARF6 and cytohesin 2 expression confirmed the requirement of both for migration of preadipocytes. Phosphatidylinositol 3-kinase (PI3K) and PI 4,5-bisphosphate (PIP_2) have also found to be essential for the cytohesin 2/ARF6 induced preadipocyte migration. Pharmacological inhibition of the activation of ARF6, ERK1/2 or dynamin led to significant reduction in migration of 3T3-L1 preadipocytes. Furthermore, our study revealed the activation of ARF6 and ERK1/2 during migration of preadipocytes. In the migrating preadipocytes, ARF6 activation was inhibited with SecinH3 (cytohesin inhibitor) and LY294002 (PI3K inhibitor) whereas the ERK1/2 phosphorylation was inhibited with SecinH3, LY294002, PBP10 (a PIP_2 sequester peptide) and PD98059 (MAPKK inhibitor). However, dynosore (dynamin inhibitor) had inhibited neither ARF6 activation nor ERK1/2 phosphorylation during preadipocyte migration. These results together suggest that cytohesin 2 activates ARF6 in a PI3K dependent manner and then the active ARF6 causes phosphorylation of ERK1/2 during preadipocyte migration.

1. Introduction

Cell migration is an important and tightly co-ordinated process for development and maintenance of tissue structures [1]. The movement of cells is controlled by spatial and temporal changes in cell membrane shape, which is driven by actin cytoskeleton [1]. The actin cytoskeleton causes morphological changes (such as filopodia, lamellipodia and actin stress fibres formation) at the plasma membrane, which are regulated by various small GTPases including ADP-ribosylation factors (ARFs) [1,2]. Preadipocyte migration is an important stage in the process of adipocyte tissue formation [3]. Adipocyte tissue formation is initiated with the proliferation of mesenchymal stem cells (MSCs), which then differentiate into immature adipocytes [3]. The preadipocytes migrate to different sites before undergoing further differentiation to form mature adipocytes, which become spherical in shape and contain lipids droplets [3]. Chronic over-nutrition of adipose tissue

Abbreviations: ARF, ADP-ribosylation factor; PI, phosphatidylinositol; PI3K, PI 3-kinase; PIP_2, PI 4,5-bisphosphate; PIP_3, PI 3,4,5-trisphosphate; ERK, extracellular-signal regulated kinase; MAPKK, mitogen-activated protein kinase kinase; PBD, protein binding domain; GTP, guanosine triphosphate; GEF, guanine nucleotide exchange factor; GAP, GTPase activating factor; BFA, Brefeldin A; EFA6, exchange factor for ARF6; BRAG, BFA resistant ARF GEF; MDCK, Madin-Darby canine kidney; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; CS, calf serum; GGA3, Golgi-associated gamma adaptin ear containing; ARF, binding protein 3; GST, glutathione S-transferase; PBS, phosphate buffered saline; IgG, immunoglubulin G; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SD, standard deviation; PH domain, pleckstrin homology domain; PKB, protein kinase B; Grp1, general receptor for phosphoinositides 1; GRSP, Grp1 signalling partner; GRASP, Grp1-associated scaffold protein; CASP, cytohesin-associated scaffold protein (CASP)/cytohesin interacting protein (CYTIP); CYTIP, cytohesin interacting protein; IPCEF1, interaction protein for cytohesin exchange factors 1.

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leads to infiltration of macrophages, which cause inflammation that inhibits insulin signalling [4].

ARFs are Ras-related small GTPases, which are about 20 kDa in size. They cycle between the active GTP bound and inactive GDP bound conformations [5]. ARFs are regulated through activation by guanine nucleotide-exchange factors (GEFs) and inactivation by GTPase-activating proteins (GAPs) [5]. There are six mammalian ARF proteins (ARFs 1–6), ARF1 and ARF6 are the best characterised and least related. ARF1 localises to and acts at the Golgi whereas ARF6 localises to and acts at the cell periphery [6]. ARF6 plays a pivotal role in cell migration by regulating actin cytoskeletal remodelling and membrane trafficking at the plasma membrane [1]. All ARFs, except ARF6, activation is inhibited by Brefeldin A (BFA), a fungal metabolite, whereas ARF6 activation by cytohesins is inhibited by a chemical inhibitor SecinH3 [7]. However, QS11, which is a cell permeable purine derivative, increases the activation of ARFs by inhibiting the ARF GAP activity [8]. There are four cytohesin family members in humans (cytohesins 1–4), each of which contain a pleckstrin homology (PH) domain that binds to PI 3,4,5-trisphosphate (PIP_3). Cytohesins 1–3 have been shown to translocate from the cytoplasm to the plasma membrane in a PI3K dependent manner, where they activate ARF6 [9–13].

Cell migration has previously been demonstrated to be regulated by ARF6 specific GEFs such as BRAGs (1–3) and exchange factor for ARF6 (EFA6) [14]. The cytohesin family of ARF GEFs have also been demonstrated in a variety of cell types to be important regulators of cell migration [15–17]. Paxillin, a focal adhesion protein, has been found to interact with and regulate cytohesin 2 activity during the migration of preadipocytes [3]. ERK1/2 and Rac1 have previously been shown to function downstream of ARF6 in cell migration [16]. The ARF6-dependent activation of ERK1/2 has been shown to be required for epithelial tubule development in Madin–Darby canine kidney (MDCK) cells and human liver cancer cell migration stimulated by epidermal growth factor (EGF) [16,18]. Dynamin has also been linked with cell migration through its involvement in regulation of the actin cytoskeleton remodelling, focal adhesion disassembly, lamellipodial accumulation and accumulation of Rac1 at the leading edge of cell during the migration [19]. In addition, the interaction between dynamin and cortactin during cell migration has previously been shown to be potentiated by ERK1/2 phosphorylation [20,21].

In addition to their role in cell migration, both cytohesin 2 and ARF6 have been implicated in early stages of insulin signalling at the insulin receptor, where inhibition of signalling led to insulin resistance in cultured hepatocytes [7,22]. The close relationship of imbalanced lipid storage and insulin resistance suggests that cytohesin 2 and ARF6 could play a role in the pathological changes observed in type 2 diabetes. Consistent with this view, we have recently shown the involvement ARF6 in endothelin-1 induced lipolysis in 3T3-L1 adipocytes [23]. In this study, cytohesin 2 and ARF6 have been found to regulate 3T3-L1 preadipocyte migration through their downstream signalling effectors ERK1/2 and dynamin. Furthermore, we have shown that cytohesin 2 activates ARF6 in a PI3K dependent manner and then the active ARF6 causes activation/phosphorylation of ERK1/2 during the migration of preadipocytes.

2. Materials and methods

2.1. Materials

Calf serum (CS) and Dulbecco's modified Eagle's medium (DMEM) were from Biosera (Uckfield, UK). Dynasore, PD98059 and SecinH3 were from Abcam (Cambridge, UK). Penetratin, ARF1p-penetratin and ARF6p-penetratin were synthesised by Thermo Fisher Scientific (Cramlington, UK). LY294002 and QS11 were from R&D Systems Europe Ltd. (Abingdon, UK). Polyphosphoinositide-Binding Peptide (PBP10) and Cell Comb Scratch Assay were from Merck Millipore Chemicals Ltd (Beeston, Nottingham, UK). Brefeldin A (BFA) was from LC Laboratories (MA, USA). Oris™ Cell Migration Assay 96-well plates were from Platypus Technologies (Madison, WI, USA). Mouse anti-Arf6 antibody was from Santa Cruz biotechnology (Santa Cruz, CA). Anti-pERK1/2 and anti-total ERK1/2 antibodies were from New England Biolabs Ltd. (Hitchin, UK). siRNA duplexes were synthesised by Eurogentec Ltd. (Southampton, UK). All other chemical unless otherwise specified were from Sigma–Aldrich (Poole, UK).

2.2. Oris cell migration assay

This assay was carried out according to the manufacturer's instructions using Oris cell migration assay kit. Briefly, 0.1 ml of 3T3-L1 preadipocytes (2.5×10^5 cells/ml) grown in DMEM containing 10% CS, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (FSM) were seeded in each test well of Oris cell migration 96-well plates and incubated the plates in a cell culture incubator for 2 h to permit cell adhesion. Cells seeded in pre-migration wells in a separate plate were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PF) for 15 min, washed again with PBS thrice and stored at 4 °C. After adding test compounds directly to cells seeded wells, the plate was incubated for a further 24 h to allow cells to migrate. The wells were then washed with PBS, fixed with 4% PF and washed again with PBS as for the pre-migration plate. After fixation, both pre-migration and post-migration wells were stained for nucleus using DAPI (1 mg/ml; 1:4000 dil.) in PBS for 10 min. The wells were washed with PBS twice for 5 min. Images of wells were captured using InCell Analyzer 2000 (GE Healthcare) and cell free area within detection zone was quantified using ImageJ software. Cell migration is presented as percentage closure of cell free zone area, calculated using the equation $([\text{pre-migration}]_{\text{area}} - \text{Oris}[\text{post-migration}]\text{area}/[\text{pre-migration}]_{\text{area}}) \times 100$.

2.3. Cell Comb Scratch Assay

Cell scratches were carried out using Cell Comb Scratch Assay. 3T3-L1 preadipocytes plated in rectangular 1-well plates were grown in FSM to confluence. After removing the medium, plates were scratched in one direction with the Cell Comb. Cells were then washed once with DMEM to remove detached cells, and incubated in 10 ml of FSM without or with inhibitors for indicated period. Cells were also incubated for 1–2 h with inhibitors, where indicated, prior to scratching. Images were captured immediately after scratching and at indicated time after scratching using a light microscope equipped with camera (Canon, EOS 600D) and cell scratch areas were quantified using ImageJ software. Cell migration in this assay is presented as percentage closure of cell scratch area, calculated using the same equation that used for Oris cell migration assay. Cells were then lysed as described in the ARF6-GTP pulldown assay and used to assess ARF6 activation and ERK1/2 phosphorylation.

2.4. ARF6-GTP pulldown assay

This assay was carried out as described previously [24]. The GST-GGA3 protein binding domain (PBD) fusion protein was purified and coupled to glutathione-Sepharose beads (GE Healthcare). Preadipocytes were washed twice in ice-cold PBS and lysed at 4 °C for 15 min with lysis buffer (500 μl per plate; 25 mM Tris–HCl [pH 7.2], 150 mM NaCl, 5 mM $MgCl_2$, 1% NP40, 5% glycerol) containing 1% mammalian protease inhibitor cocktail (Sigma Aldrich, Poole, UK). The cell lysates were centrifuged at 14,000 × *g*

for 10 min at 4 °C to pellet cellular debris. A 400 μl fraction of each lysate was incubated with glutathione-Sepharose beads coupled to 25 μg of purified GST-GGA3 PBD at 4 °C for 2 h. The beads were washed three times with wash buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, and 1% Triton X-100) and boiled in 50 μl 1× SDS-PAGE loading buffer for 5 min to release the bound protein into the buffer. The lysates that were not incubated with the beads were used to assess total ARF6 and ERK1/2 phosphorylation (ERK1/2-p) [23]. ARF6-GTP bound to the beads and total ARF6, total ERK1/2 and ERK1/2-p in the inputs were determined by immunoblotting using anti-ARF6 (1:200 dil.), anti-ERK1/2 (1:1000 dil.) and anti-phospho ERK1/2 (1:1000 dil.) antibodies.

2.5. Immunoblotting

Proteins were separated by 12% SDS-PAGE, transferred onto PVDF membrane and immunoblotted using primary antibodies and the HRP-conjugated secondary antibodies as described earlier [25,26].

2.6. siRNA transfection

The specific target nucleotide sequence of ARF1 siRNA, ARF6 siRNA and cytohesin 2 siRNA was described previously [3,24]. The siRNA duplex consisting of a unique sequence that does not have significant homology to any mammalian gene sequences was used as a negative control. Transfection of 3T3-L1 cells with siRNA was performed when the cells had reached 30–50% confluency. For a 6 cm^2 plate, 10 μl Lipofectamine RNAiMAX was diluted in 500 μl of OPTIMEM. To this, 100 nM siRNA was added. The resulting mixture was incubated for 15 min at room temperature and then added to the cells growing in FSM without antibiotics. After 4 h of incubation, the medium was changed to FSM and left for a further incubation of 44 h and then used for the cell migration assays and immunoblotting.

2.7. Inhibition of ARF activation through penetratin-bound inhibitory peptides

The cell-permeating domain of *Drosophila* antennapedia protein (penetratin) and the N-myristoylated ARF1 (ARF1p; consists of 2–17 aa of ARF1) and ARF6 (ARF6p; consists of 2–13 aa of ARF6) peptides fused to penetratin, to give the property of membrane permeability, were used in this assay [23,24]. 5 μM penetratin, 1.5–10 μM ARF1p-penetratin or 1.5–10 μM ARF6p-penetratin were added to designated wells during the cell migration assay.

2.8. Statistical analyses

As described in Oris cell migration and Cell Comb Scratch Assays, preadipocyte migration was quantified using ImageJ software. Data presented as percentage closure of cell free zone (Oris cell migration assay) or scratch (Cell Comb Scratch Assay) was subsequently analysed using the statistical analysis software programme, GraphPad Prism. The results are presented as means ± standard deviation (SD). Statistical significance was assessed by one-way ANOVA. A P value <0.05 was considered as statistically significant.

3. Results

3.1. ARF6 and cytohesin 2 are involved in the migration of 3T3-L1 preadipocytes

Cytohesin 2, ARF1 and ARF6 have recently been shown to be involved in 3T3-L1 preadipocyte migration [3]. To confirm the role of these proteins in preadipocyte migration, we first studied the effect of down regulation of ARF1, ARF6 and cytohesin 2 expressions on preadipocyte migration using previously validated cytohesin 2, ARF6 and ARF1 siRNAs [3,24]. The specific down regulation of target proteins using siRNA was assessed by immunoblotting (Fig. 1A). ARF1 expression was significantly reduced (approximately 80%) in ARF1 siRNA transfected cells but not in either ARF6 or cytohesin 2 or negative control siRNA transfected cells. Similarly ARF6 and cytohesin 2 expression was considerably depleted (approximately 90%) only in the cells transfected with ARF6 and cytohesin 2 siRNAs respectively. Depletion of ARF6 and cytohesin 2 by siRNA treatment significantly reduced migration of preadipocytes as assessed by the Oris cell migration assay (Fig. 1B). As observed previously [3], the reduction in ARF1 protein expression had the least effect on preadipocyte migration (76.46% ± 6.145 vs. 87.75% ± 6.374 in control, $P < 0.05$). ARF6 protein depletion resulted in a greater reduction in preadipocyte migration (57.71% ± 5.328 vs. control, $P < 0.001$). The greatest reduction in preadipocyte migration was observed in cells with down regulation of cytohesin 2 protein expression (45.31% ± 6.171 vs. control, $P < 0.001$).

3.2. Penetratin coupled ARF6p preferentially inhibits 3T3-L1 preadipocyte migration

To confirm the preferential involvement of ARF6 in the migration of 3T3-L1 preadipocytes, the inhibitory peptides of ARF1 and ARF6 (ARF1p and ARF6p) were utilised [24,27,28]. ARF1p and ARF6p peptides were coupled to the cell-permeating domain of *Drosophila* antennapedia protein (penetratin) to make them membrane permeable. As shown in Fig. 2A, penetratin had no effect on 3T3-L1 preadipocyte migration whereas ARF1p-penetratin and ARF6p-penetratin inhibited preadipocyte migration. However, ARF6p-penetratin was more efficient than ARF1p-penetratin in inhibiting preadiopcyte migration. Both penetratin coupled ARF1p (Fig. 2B) and ARF6p (Fig. 2C) inhibited migration of preadipocytes in a dose-dependent manner but greater inhibition was observed in preadipocytes exposed to ARF6p-penetratin. ARF1p-penetratin had no effect on preadipocyte migration at 1.25 μM (92.72% ± 5.480 vs. 96.81% ± 2.432 in control) but showed inhibition at higher dose, the maximal affect was at 10 μM dose (68.23% ± 3.653 vs. 96.81% ± 2.432 in untreated control, $P < 0.001$). However, ARF6p-penetratin significantly inhibited preadipocyte migration at 1.25 μM (59.38% ± 3.423 vs. 84.90% ± 4.6 in control, $P < 0.001$). Inhibition of migration increased with increasing dose to 10 μM (41.15% ± 3.653 vs. control, $P < 0.001$). These observations, like those seen in the case for siRNA treatment, highlighted the preferential involvement of ARF6 in migration of 3T3-L1 preadipocytes. This corresponds with the greater role for ARF6 over ARF1 in preadipocyte migration described earlier [3].

3.3. ARF6 and ERK1/2 are activated during 3T3-L1 preadipocyte migration following scratching

A previous study revealed successive activation of ARF6 and ERK1/2 during migration of hepatoma cells [16]. Since ARF6 is required for preadipocyte migration, we hypothesised ARF6 activation during preadipocytes migration. As the wells of Oris cell migration plates we used are of a 96-well format, the Cell Comb Scratch Assay in 1-well format was employed to scale up the migration assay to assess biochemical events during preadipocyte cell migration. We first assessed cell migration using the Cell Comb Scratch Assay. As shown in Fig. 3A, preadipocytes have migrated 68.49% width of the scratch within 4 h. Unlike in the Oris cell migration assay, the scratch closed completely within 24 h in the Cell Comb Scratch Assay. This is because the diameter of cell free

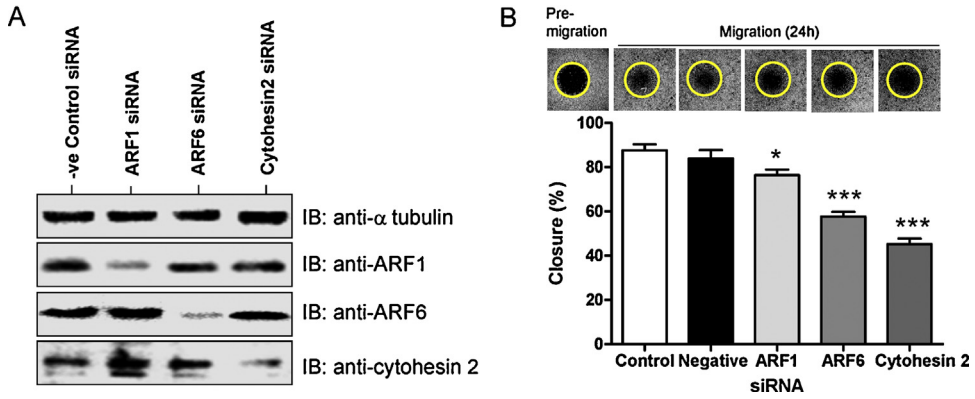

Fig. 1. Migration of 3T3-L1 preadipocytes is affected by cytohesin 2 and ARF6 depletion. 3T3-L1 preadipocytes transfected with siRNA for either negative control, ARF1, ARF6 or cytohesin 2 for 48 h were subjected to either immunoblotting (A) or Oris Pro Cell Migration assay (B). (A) The siRNA transfected cells were lysed and 30 μg protein of each sample was fractionated by SDS-PAGE, immunoblotted with anti-tubulin, anti-ARF1, anti-ARF6 or anti-cytohesin 2 primary antibodies and down regulation of the target protein was assessed by scanning the blots. (B) Oris cell migration assay wells were seeded with siRNA transfected 3T3-L1 preadipocytes and incubated for 0 h (pre-migration control) or 24 h to permit cell migration. Then the cells were fixed with 4% paraformaldehyde and stained with DAPI. Migration of 3T3-L1 preadipocytes into cell free zone (marked by a circle) was determined using the InCell Analyzer 2000 (*upper panel*), images processed with ImageJ software and cell migration is presented as percentage closure of the cell free zone (*lower panel*). The percentage of cell free zone closure done in duplicate is displayed as means ± SD of 3 separate experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with no treatment.

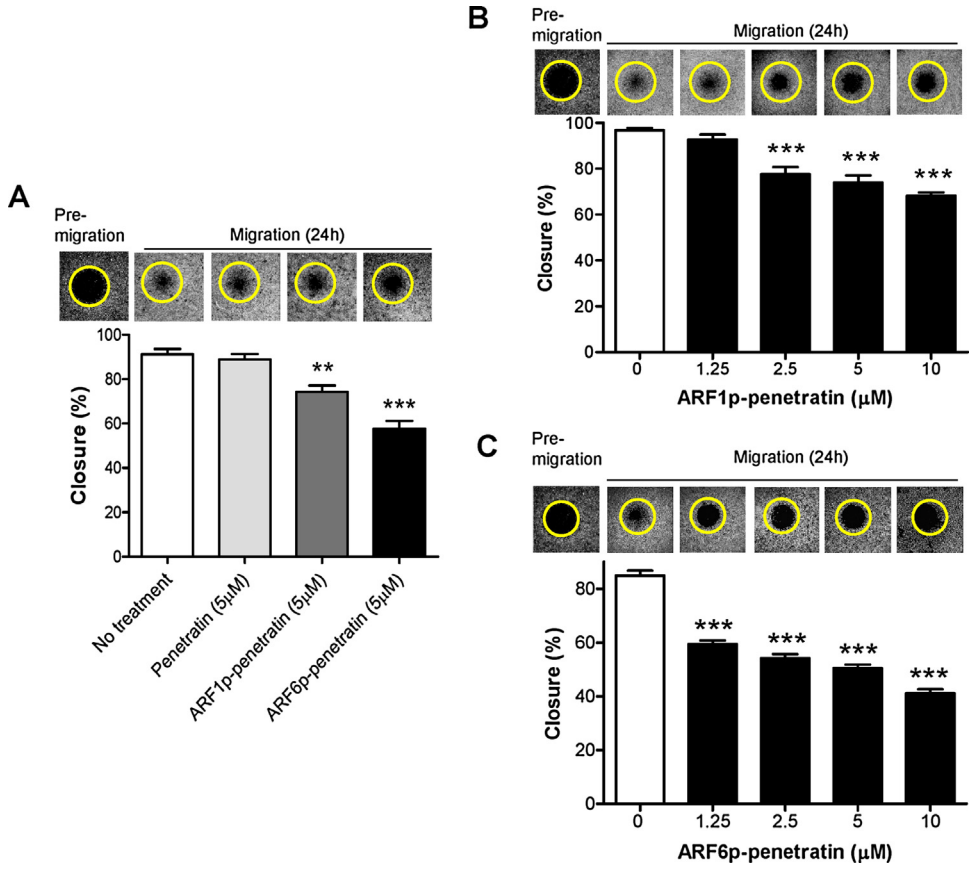

Fig. 2. 3T3-L1 preadipocyte migration is inhibited preferentially by penetratin coupled ARF6 inhibitory peptide. The Oris Pro Cell Migration assay wells seeded with 3T3-L1 preadipocytes were incubated independently with 5 μM penetratin, 5 μM ARF1p-pentratin and 5 μM ARF6p-penetratin (A) or varying concentrations (1.25–10 μM) of ARF1p-penetratin (B) and ARF6p-penetratin (C) for 24 h to permit cell migration. At the end of migration, the cells were fixed with 4% paraformaldehyde and stained with DAPI. Migration of 3T3-L1 preadipocytes was determined as described in Fig. 1. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with no treatment.

zone in Oris cell migration plate is 2.5 mm whereas the width of cell free zones obtained following the cell comb scratch is about 0.2 mm. We next assessed whether ARF6 and ERK1/2 get activated during cell migration using the Cell Comb Scratch Assay (Fig. 3B/C & 4). As shown in Fig. 3B, ARF6 was activated within 5 min after scratching and the ARF6-GTP levels were maintained between 5.4-fold (5 min) and 5.7-fold (240 min) relative to that in unscratched cells (basal levels) during the time course of the scratch assay. ERK1/2 was also activated (through phosphorylation) by scratching but the activation occurred later (30 min after the scratching) when compared to ARF6 activation (Fig. 3 C). Similar to the ARF6 activation, ERK1/2 activation was maintained between 6.5-fold (30 min) and 6.1-fold (240 min) relative to that in unscratched cells. However, we did not observe ARF6 and ERK1/2

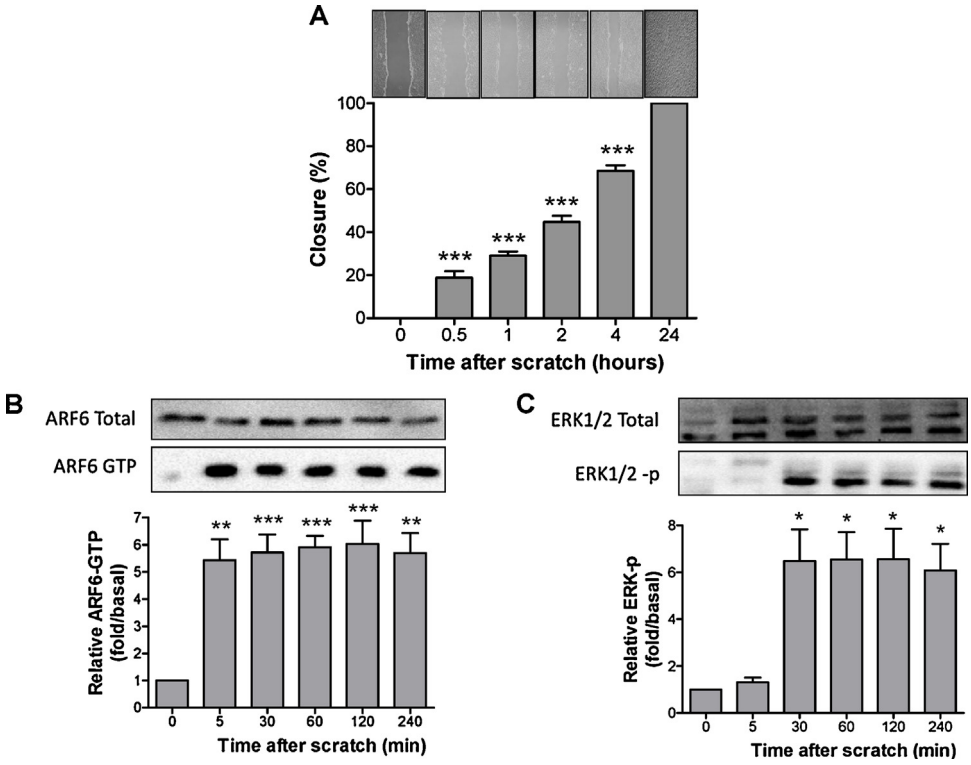

Fig. 3. Assessment of time-dependent migration of 3T3-L1 preadipocytes and the activation of ARF6 and ERK1/2 during preadipocytes migration by Cell Comb Scratch Assay. 3T3-L1 preadipocytes were grown to confluent cell monolayers were scratched using the cell comb and incubated for 0–24 h to allow migration of preadipocytes. After that time, preadipocyte monolayers were viewed under phase contrast microscopy (10×), images were captured using a camera (A, *upper panel*), processed with ImageJ software and cell migration is presented as percentage closure of the cell scratch area (A, *lower panel*). The percentage of cell scratch area closure is displayed as means ± SD of three separate experiments. $^{***}P < 0.001$ compared with scratch at 0 h.3T3-L1 preadipocytes grown in 1-well plates to confluent cell monolayers were scratched using the cell comb and incubated in fresh medium for 0–240 min to allow migration of preadipocytes (B & C). The cells in scratched plates at each time point of incubation and a control plate of confluent preadipocytes were then lysed and incubated the lysates with GST-GGA3 PBD resin to precipitate active ARFs. The protein bound to the resin was analysed by immunoblotting using an anti-ARF6 antibody to assess the levels of ARF6-GTP. The cell lystes that not incubated with the GST-beads were also immunoblotted with an anti-ARF6 antibody, an anti-ERK antibody and an anti-ERKphospho specific antibody for analysing total ARF6, total ERK1/2 and the phosphorylated ERK1/2 (ERK1/2-p) respectively. Densitometric analysis of ARF6-GTP (B, *lower panel*) and ERK1/2-p (C, *lower panel*) is shown as a histogram after normalising them to the levels of total ARF6 (B, *upper panel*) and ERK1/2 (C, *upper panel*), respectively, in the sample. Values are means ± SD of three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with scratch at 0 h.

activation above the basal levels after complete closure of the scratch (24 h after scratching) by preadipocytes migration (Fig. 4). This result revealed the activation of ARF6 and ERK1/2 during migration of 3T3-L1 preadipocytes.

3.4. Cytohesin 2 ARF GEF regulate 3T3-L1 preadipocyte migration

The depletion of cytohesin 2 has been shown to prevent the activation of ARF6, but not ARF1, during preadipocyte migration, indicating that ARF6 is the substrate for cytohhesin 2 GEF activity [3]. To determine the role of cytohesin 2 and ARF6 in preadipocyte migration, we first studied the effect of various concentrations (6.25–50 μM) of SecinH3, a chemical inhibitor for the ARF6 activation by cytohesins, on 3T3-L1 preadipocyte migration over 24 h [7]. SecinH3 inhibited the migration significantly at 6.25 μM (53.65% ± 3.072 vs. 87.50% ± 5.929 in control, $P < 0.001$) and maximum at 50 μM (38.02% ± 6.379 vs. control, $P < 0.001$) (Fig. 5A). At 50 μM SecinH3, more than 60% of migration of 3T3-L1 preadipocytes was inhibited. This result indicates the involvement of cytohesin 2 in the migration of 3T3-L1 preadipocytes through ARF6 activation [3].

3.5. Inhibition of ARF6 inactivation increases migration of 3T3-L1 preadipocytes

To further assess the requirement of ARF6 activation in 3T3-L1 preadipocyte migration, an ARF GAP inhibitor (QS11) was used to prevent ARF6 inactivation during migration. QS11, a small purine derivative is a broad spectrum ARF GAP inhibitor [8]. A dose dependent effect of QS11 (6.25–50 μM) on preadipocyte migration was tested. As shown in Fig. 5B, migration of preadipocytes was unaffected by 6.25 μM and 12.5 μM of QS11. However, there was a slight increase in preadipocyte migration in the presence of 25 μM QS11 (89.58% ± 3.48 vs. 80.73% ± 6.379 in control, $P < 0.01$) and the migration slightly increased further with 50 μM QS11 treatment (91.77% ± 4.097 vs. 80.73% ± 6.379 in control, $P < 0.001$). This result indicates further that the GTP-bound ARF6 is important for preadipocyte migration.

3.6. PIP_2 and PI3K are essential effectors for 3T3-L1 preadipocye migration

A recent study revealed the necessity of second messenger PIP_3, which produced by PI3K, in the recruitment of cytohesin 2 to the plasma membrane during cell migration and the membrane recruited cytohesin 2 for activation of ARF6 [29]. We have also previously shown the PI3K-dependent translocation of cytohesins 1–3 from the cytosol to the plasma membrane, where they activate ARF6 [9–13]. To investigate the role of PIP_3 in migration of 3T3-L1 preadipocytes, a chemical inhibitor of PI3K (LY294002) was used. LY294002 is a reversible and selective inhibitor of PI3K with the advantage of an increased half-life in comparison to the other PI3K inhibitors such as wortmannin

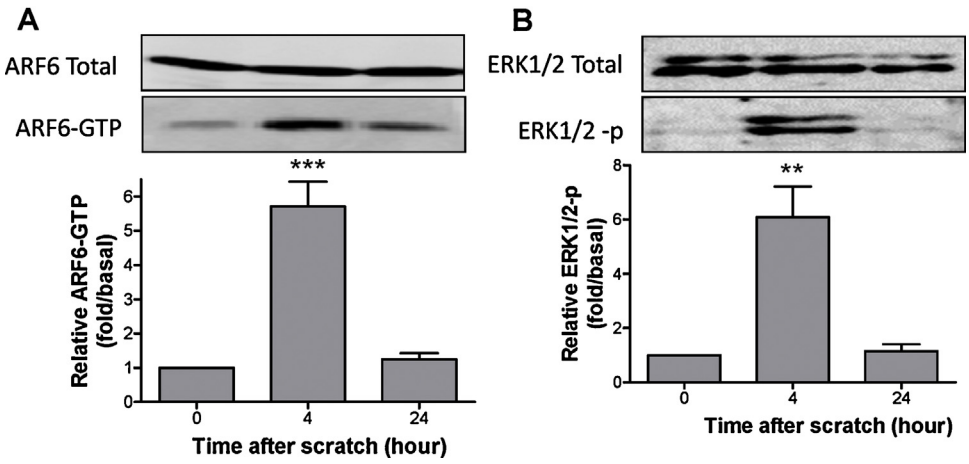

Fig. 4. Time-dependent activation of ARF6 and ERK1/2 during 3T3-L1 preadipocyte migration in Cell Comb Scratch Assay. 3T3-L1 preadipocytes grown to confluent cell monolayers were scratched using the cell comb and incubated for 4 h or 24 h to allow migration of preadipocytes. Cells of plates scratched at each time point and control plates of confluent preadipocytes were then lysed and subjected to the GST-GGA3 PBD pulldown assay. Total ARF6, ARF6-GTP, total ERK1/2 and ERK1/2-p were analysed by immunoblotting. The blots are representative of three separate experiments. Densitometric analysis of ARF6-GTP (A, *lower panel*) and ERK1/2-p (B, *lower panel*) is shown as a histogram after normalising them to the levels of total ARF6 and total ERK1/2, respectively, present in the sample. Values are means ± SD of three separate experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with scratch at 0 min.

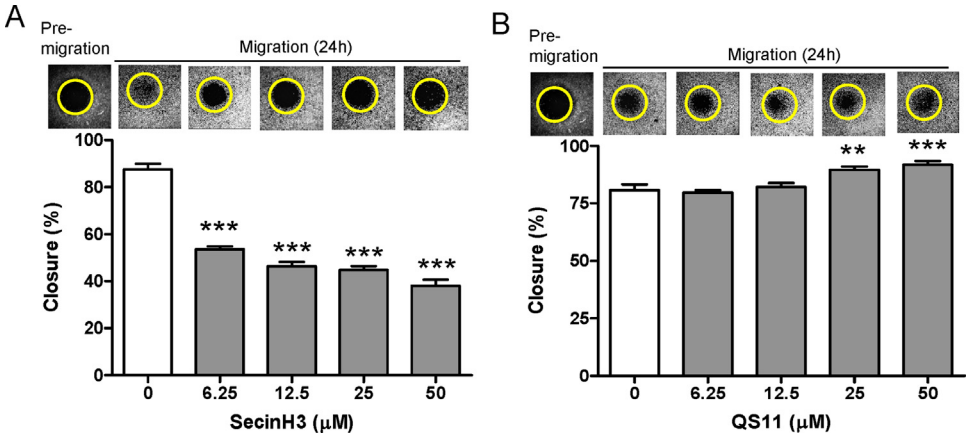

Fig. 5. 3T3-L1 preadipocyte migration signals through cytohesin 2, ARF6, ERK1/2 and dynamin. The Oris Pro Cell Migration assay wells seeded with 3T3-L1 preadipocytes were incubated for 24 h to permit cell migration in the presence of varying concentrations (6.25–50 μM) of the cytohesin inhibitor SecinH3 (A) or ARF GAP inhibitor QS11 (B). After that, the cells were fixed with 4% paraformaldehyde and stained with DAPI. Migration of 3T3-L1 preadipocytes was determined as described in Fig. 1. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with no treatment.

[30]. Inhibition of PI3K prevents the formation of second messenger PIP_3, which subsequently inhibits cytohesin 2 recruitment to the plasma membrane and thereby the activation of ARF6. ARF6-GTP binds and activates PI 4-phosphate 5-kinase (PIP5K), leading to a large increase in PIP_2 levels at the cell surface [31]. ARF6-GTP and PIP_2 function synergistically to recruit AP-2 (a component of the clathrin coat complex), suggesting a role for ARF6 in clathrin mediated internalisation essential for cell migration [24]. PIP_2 sequestering peptide PBP10, which is a decapeptide derived from the PIP_2-binding region in segment 2 of gelsolin [32], was used to study the role of PIP_2 in preadipocyte migration. As shown in Fig. 6, sequestering of PIP_2 with PBP10 resulted in significant inhibition of preadipocyte migration (53.13% ± 4.419 vs. 83.05% ± 3.429 in control, $P < 0.01$). Pharmacological inhibition of PI3K by LY294002 also led to significant reduction in preadipocyte migration (40.94% ± 3.977 vs. control, $P < 0.001$). These observations highlight the importance of PIP_2 and PI3K in preadipocyte migration. BFA, an ARF GEF inhibitor that does not prevent the ARF6 activation, did not affect preadipocyte migration (Fig. 6).

3.7. ERK1/2 and dynamin play a role in preadipocytes migration

Hu and colleagues revealed ERK1/2 and Rac1 activation following ARF6 activation at the plasma membrane during cell migration [16]. ARF6 dependent activation of ERK1/2 and Rac1 in epithelial tubule development was also described [18]. Dynamin is an important GTPase in the process of cell migration, where it regulates focal adhesion disassembly to allow detachment from surrounding cells [19]. Dynamin, along with Rac1, also regulates cell migration through participation in extracellular matrix degradation and lamellipodial accumulation at the leading edge of the cell [19].

To determine the role of ERK1/2 and dyanamin in preadipocyte migration, chemical inhibitors PD98059 and dynasore were used. PD98059 is a specific inhibitor for the phosphorylation of ERK1/2 by mitogen-activated protein kinase kinase (MAPKK). Dynasore is a small molecule inhibitor of dynamin [24]. As shown in Fig. 7A, inhibition of ERK1/2 phosphorylation with 12.5 μM PD98059 resulted in significant inhibition of preadipocyte migration (62.03% ± 9.667 vs. 82.34% ± 5.859 in control, $P < 0.01$). Migration

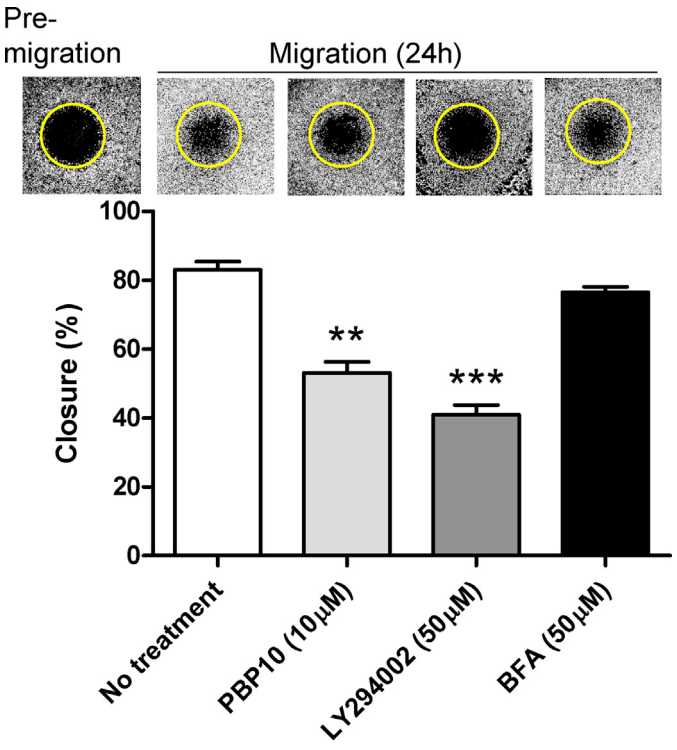

Fig. 6. Inhibition of PI3K and depletion of PIP_2 reduced 3T3-L1 preadipocyte migration. The Oris Pro Cell Migration assay wells seeded with 3T3-L1 preadipocytes were treated with PIP_2 sequestering peptide PBP10 (10 μM), the PI3K inhibitor LY294002 (50 μM) or an ARF GEF inhibitor BFA (50 μM) and incubated for 0 h (pre-migration control) or 24 h to permit cell migration. At the end of cell migration, the cells were fixed with 4% paraformaldehyde and stained with DAPI. Migration of 3T3-L1 preadipocytes was determined as described in Fig. 1. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with no treatment.

of preadipocytes decreased with increasing dose of PD98059, reaching maximal inhibition at 50 μM (46.85% ± 9.917 vs. control, $P < 0.001$). At 50 μM of PD98059, migration of 3T3-L1 preadipocytes was reduced by almost half that of the control, confirming ERK1/2 involvement in preadipocyte migration. As shown in Fig. 7B, inhibition of dynamin with dynasore also reduced the migration of preadipocytes. At 6.25 μM dynasore, migration was significantly inhibited (52.08% ± 4.27 vs. 85.42% ± 7.569 in control, $P < 0.001$) and the inhibition increased further with increasing concentration to 50 μM (28.23% ± 9.441 vs. control, $P < 0.001$). At 50 μM dynasore, migration of preadipocytes was reduced to less than a third that of the control. Taken together, these results demonstrate the importance of ERK1/2 and dynamin in preadipocyte migration. Like in Oris cell migration assay, SecinH3, PD98059 and dynasore also inhibited preadipocyte cell migration after scratching, indicating that the results obtained using the Cell Comb Scratch Assay are comparable with those of Oris cell migration assay (Fig. 8). When we assessed cell viability using an alamar blue assay, none of inhibitors at the highest concentration used in cell migration assay had any effect on preadipocytes viability (data not shown). This indicates that the effect of inhibitors used in this study on preadipocytes migration is specific.

3.8. ERK1/2 and dynamin are downstream signalling effectors of ARF6

Since inhibition of cytohesin, MAPKK and dynamin lead to slowdown of 3T3-L1 preadipocyte migration, we further investigated whether these signalling molecules regulate cell migration by altering the activity of ARF6 and ERK1/2. For this purpose, the cell migration inhibitors effect, at an optimal concentration, on the activation of ARF6 and ERK1/2 during preadipocytes migration was assessed. As shown in Fig. 9A, there was a 5-fold increase in ARF6-GTP levels during 240 min cell migration. In the presence of SecinH3 (a cytohesin inhibitor) or LY294002 (a PI3K inhibitor), cell migration-induced ARF6-GTP was reduced to basal levels, which correlated with the observed inhibition of preadipocyte migration. Treatment of preadipocytes with QS11 (an ARF GAP inhibitor) did slightly increased the ARF6-GTP levels (5.7-fold). However BFA (an ARF GEF inhibitor that does not prevent ARF6 activation), PD98059 (a MAPKK inhibitor), PBP10 (a PIP_2 sequestering peptide) and dynasore (a dynamin inhibitor) had no effect on the cell migration-induced activation of ARF6.

As shown in Fig. 9B, there was a 6.4-fold increase in ERK1/2-phosphorylation/activation during 240 min of cell migration. SecinH3 inhibited the ERK1/2 activation (2.2-fold vs. 6.4-fold in control) whereas QS11 increased the ERK1/2 activation to 8.9-fold, the highest levels of ERK1/2-phosphorylation in the assay. As seen with the ARF6 activation, BFA and dynasore had no effect on the cell migration-induced activation of ERK1/2. However, PD98059 inhibited ERK1/2 activation (2.1-fold vs. 6.4-fold in control), which correlated with the inhibition of migration observed with PD98059 treatment. In the presence of PBP10 or LY294002, ERK1/2

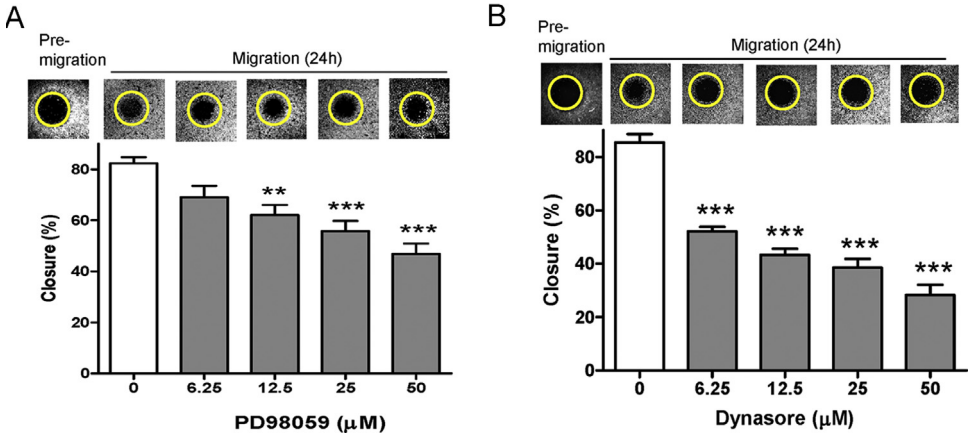

Fig. 7. 3T3-L1 preadipocyte migration signals through ERK1/2 and dynamin. The Oris Pro Cell Migration assay wells seeded with 3T3-L1 preadipocytes were incubated for 24 h to permit cell migration in the presence of varying concentrations (6.25–50 μM) of the MEKK inhibitor PD98059 (A) or dyanmin inhibitor dynasore (B). After that, the cells were fixed with 4% paraformaldehyde and stained with DAPI. Migration of 3T3-L1 preadipocytes was determined as described in Fig. 1. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with no treatment.

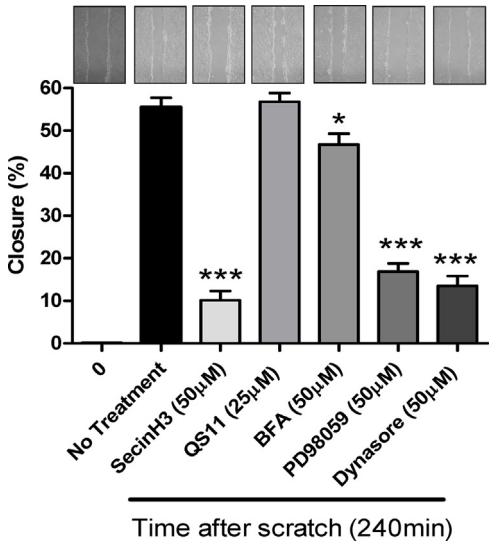

Fig. 8. Inhibition of cytohesin, ERK1/2 and dynamin activation affects migration of 3T3-L1 preadipocytes in Cell Comb Scratch Assay. 3T3-L1 preadipocytes grown to confluent cell monolayers in 1-well plates were incubated without or with inhibitors for 1 h, scratched using the cell comb and incubated again without or with inhibitors for 4 h to allow migration of preadipocytes. After that time, preadipocyte monolayers were viewed under phase contrast microscopy (10×) and images were captured using a camera (*upper panel*), processed with ImageJ software and cell migration is presented as percentage closure of the cell scratch area (*lower panel*). The percentage of cell scratch area closure is displayed as means ± SD of three separate experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with no treatment.

phosphorylation was also reduced to basal levels, correlating with the observed inhibition of preadipocyte migration. These results demonstrate that preadipocyte migration signals through the PI3K and cytohesin ARF GEF mediated activation of ARF6, followed by that of ERK1/2. This is suggested as PD98059 inhibited preadipocyte migration and ERK1/2 activation during the migration but had no effect on the activation of ARF6. Previously we have shown that ARF6 act upstream of dynamin, by activating it through NM23-H1, and clathrin, by generating PIP_2 through the activation of PIP5K [24]. Dyanmin has also shown to act down stream of ERK1/2 [20,21,33]. Dynasore and PBP10 had no effect on cell migration-induced ARF6 activation though they inhibit preadiocyte migration, indicating that dynamin and PIP_2 are also likely to act downstream of ARF6 in cell migration. Like the ARF6 activation, cell migration-induced ERK1/2 activation was also unaffected by dynasore and this suggests that dynamin may act downstream of ARF6 and ERK1/2. However, ERK1/2 activation during cell migration was affected by PBP10, suggesting that PIP_2 may act upstream of ERK1/2 phosphorylation.

4. Discussion

The involvement of ARF6 in preadipocyte migration has recently been described [3]. In this study we have shown that inhibition of ARF6 activation by SecinH3 results in decreased migration of 3T3-L1 preadipocytes. In addition, prevention of ARF6 inactivation with an ARF GAP inhibitor (QS11) slightly increases preadipocyte migration, highlighting further the requirement of active, GTP-bound, ARF6 for cell migration. Consistent with this, the siRNA-mediated down regulation of ARF6 and cytohesin 2 proteins expression in preadipocytes results in a substantial reduction in migration of preadipocytes. ARF6 involvement in 3T3-L1 preadipocyte migration is also confirmed by using the membrane-permeable penetratin-coupled ARF6p inhibitory peptide. Taken together these results confirm a previous study [3] that 3T3-L1 preadipocyte migration is regulated through the activation of ARF6 by cytohesin 2. To complement the cell migration studies, the activation of ARF6 during cell migration was biochemically analysed.

The second messenger PIP_3, generated by PI3K, recruits cytohesins, through their PH domains, to the plasma membrane, where they regulate ARF6 activation [9,34–36]. It is well known that the activated ARF6 causes several morphological changes at the plasma membrane, including the formation of cortical actin [1,12,37]. The requirement of PIP_3 for the recruitment of

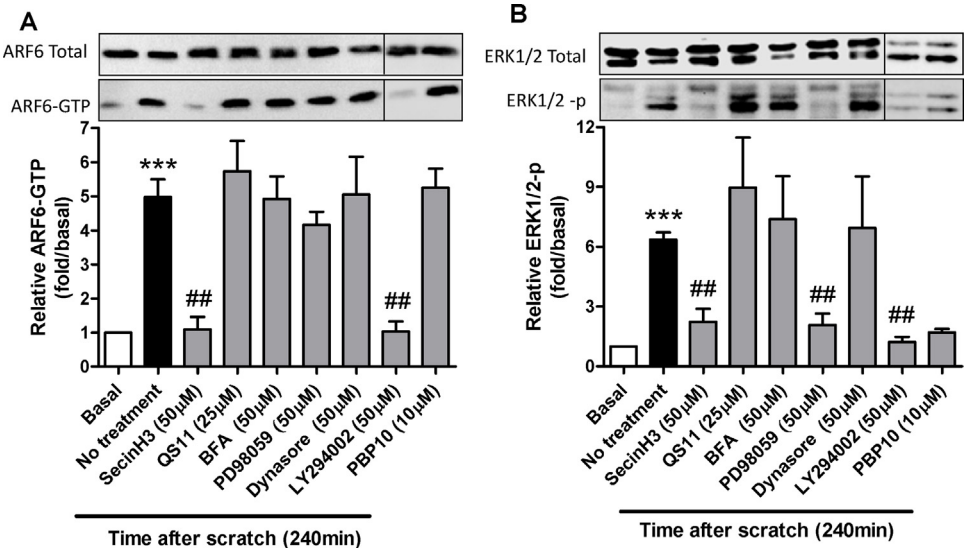

Fig. 9. Inhibition of cytohesin, ERK1/2 and dynamin activation prevents migration of 3T3-L1 preadipocytes in Cell Comb Scratch Assay. 3T3-L1 preadipocytes grown in 1-well plates to confluent cell monolayers were preincubated without or with inhibitors for 1 h, scratched using the cell comb and then incubated without and with inhibitors for 4 h to allow migration of preadipocytes. Cells of plates for each treatment and control plates of confluent preadipocytes were then lysed and subjected to the GST-GGA3 PBD pulldown assay. Total ARF6, ARF6-GTP, total ERK1/2 and the phosphorylated ERK1/2 (ERK1/2-p) were analysed by immunoblotting. The blots are representative of three separate experiments. Densitometric analysis of ARF6-GTP (A, *lower panel*) and ERK 1/2-p (B, *lower panel*) is shown as a histogram after normalising them to the levels of total ARF6 (A, *upper panel*) and ERK1/2 (B, *upper panel*), respectively, present in the sample. Values are means ± SD of three separate experiments. $^{***}P < 0.001$ compared with basal. ## $P < 0.01$ compared with scratch/no treatment.

cytohesins 2 and 3 to the plasma membrane during migration of breast carcinoma SUM159 cells has recently been demonstrated [29]. In this study, 3T3-L1 preadipocyte migration and ARF6 activation during the migration were shown to be inhibited by the PI3K inhibitor (LY294002), indicating cytohesin 2 recruitment to the plasma membrane during cell migration is through PIP_3.

In MDCK cells, ARF6 activated during HGF induced tubule development has been found to signal through ERK1/2 and Rac1 [18]. More recently, ARF6 has been shown to regulate EGF induced human hepatoma cell migration by signalling through downstream activation of ERK1/2 and Rac1 [16]. Rac1 has been shown to be accumulated with dynamin at the leading edge of the cell during migration, where it regulates cellular events such as actin cytoskeleton reorganisation [19]. ERK1/2 phosphorylation has been shown to stimulate the interaction of dynamin with cortactin, leading to the actin cytoskeleton remodelling responsible for cell migration [20,21]. These studies highlight the involvement of ERK1/2, dynamin and Rac1 in ARF6-mediated actin remodelling during cell migration.

The involvement of ERK1/2 and dynamin in preadipocyte migration demonstrated here could possibly be the link between ERK1/2 and Rac1 in actin cytoskeleton reorganisation at the plasma membrane in the process [19]. It is also possible that dynamin might be the stage at which pathways diverge following the ARF6 activation in cell migration. Dynamin, which participates in fission of clathrin-coated vesicles, is activated by ARF6-GTP through recruiting NM23-HI, a dynamin activator, to the membrane [24]. Recently, PIP_2 and clathrin have been shown to participate in cell migration. The AP2, which is a part of clathrin coat complex, interacts with PIP_2 at the cell membrane to initiate cell migration [38]. In this study, PIP_2 sequestering abolished cell migration and ERK1/2 phosphorylation but had no effect on ARF6 activation, confirming the involvement of PIP_2 downstream of ARF6 and upstream of ERK1/2 in cell migration. This is consistent with a previous study, which suggested PIP_2 regulation of ERK1/2 activation during stress induced apoptosis [33]. Although how PIP_2 regulates ERK1/2 activation is unclear, it is possible that the regulation could involve PI mediated regulation of Raf, which regulates ERK1/2 activation through the MAPK pathway [39].

Preadipocyte migration is important for lipid metabolism. Reduced preadipocyte migration through inhibition of ARF6/cytohesin2 could have negative consequences on the storage of cellular lipids. Moreover, adipocyte over-nutrition as a result of perturbed preadipocyte migration is potentially a key factor in the initiation of insulin resistance [4]. There remains the possibility of other GEFs such as BRAGs 1–3 and EFA6 acting on ARF6 during the process of migration [14]. However, there is no evidence for these GEFs expression in preadipocyte. In addition to PIs, scaffolding protein paxillin plays an important role in the recruitment of cytohesin to the plasma membrane during cell migration [3]. Scaffolding proteins such as cytohesin 3/Grp1 signalling partner (GRSP) [34], Grp1-associated scaffold protein (GRASP)/tamalin [40,41], cytohesin-associated scaffold protein (CASP)/cytohesin interacting protein (CYTIP) [42,43] and interaction protein for cytohesin exchange factors (IPCEF)1 [44] are also involved in cytohesin recruitment. IPCEF1 and GRASP have also been found to promote signalling of cytohesin 2 and Rac GEF Dock 180 during migration of MDCK cells [45]. It has been suggested that GRASP and IPCEF1 are responsible for recruitment of cytohesin 2 to the plasma membrane and thereby promoting ARF6 activation, which in turn recruits the Dock180/ELMO complex for activation of Rac1 at the plasma membrane [45]. It is possible that different combinations of ARF6 GEFs and Rac1 GEFs act together to induce downstream membrane remodelling events in different cell types. Therefore, more work regarding this aspect would yield a greater detail of the molecular mechanisms underlying preadipocyte migration. ERK1/2 has shown to be involved in ARF6 induced activation of Rac1 during EGF induced migration of HepG2 cells [16]. It is therefore possible that ERK1/2 may also act as an intermediate between ARF6 and Rac1 in preadipocyte cell migration.

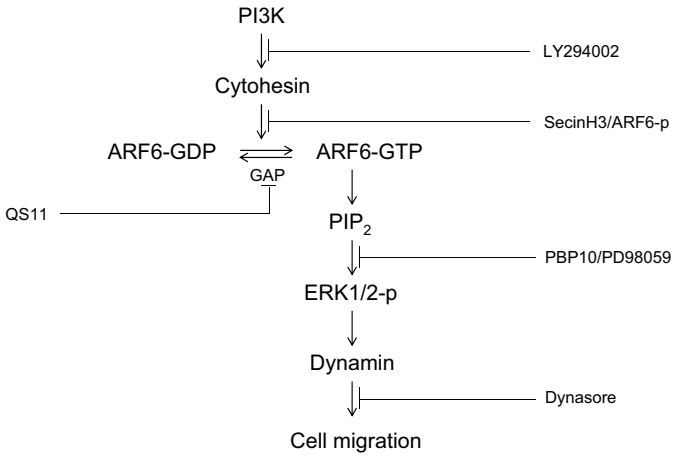

Fig. 10. Schematic model representing the ARF6 signalling pathway in migration of preadipocytes deduced from the present study.

Cytohesin 2 and ARF6 are also key signalling components of the insulin receptor, where inhibition of these effectors causes blocking of insulin signalling and thereby insulin resistance [7,22]. Therefore, in addition to adipocyte over-nutrition, the imbalance between insulin signalling and migration of the preadipocyte could also play a role in insulin resistance. These pathways and the signalling molecules common to both the pathways are of particular interest, considering the close association between obesity and insulin resistance. For instance in the case of hyperinsulinaemia, there could be a possible competition between these two signalling pathways, where increased insulin signalling through the insulin receptor recruits cytohesins and ARF6 to the receptor, causing an imbalance favouring redistribution of the signalling components from the leading edge of the preadipocyte. Further elucidation of molecular mechanisms could yield significant findings, leading to therapeutics for insulin resistance and type 2 diabetes. In summary, we proposed a pathway for ARF6/cytohesin 2 mediated preadipocyte migration based on this study (Fig. 10), cytohesin 2 recruits, by binding to PI3K lipids product (PIP_3), to the plasma membrane (PM), where it activates ARF6. The active ARF6 then increases PIP_2 levels, which leads to phosphorylation of ERK1/2 and subsequent activation of dynamin, causing actin cytoskeleton remodelling beneath the plasma membrane that required for cell migration.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2014.09.023.

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